IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the patent application of: Tachibana

Serial No. 10/551,469 Group Art Unit 1642 Conf. 3 4441

Filed 06/06/2006 Examiner: Davis

For: METHOD OF SCREENING DRUG WITH THE USE OF 67 kDa LAMININ RECEPTOR AND DRUG OBTAINED THEREBY

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF HIROFUMI TACHIBANA

Hirofumi Tachibana declares as follows:

1. I am the inventor of the claimed subject matter of the above-identified United States patent application. I currently hold the position of Associate Professor in the Division of Applied Biological Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, at Kyushu University in Japan. My research is carried out in the Laboratory of Functional Food Design, Department of Functional Metabolic Design at the Bio-Architecture Center at Kyushu University.

2. The data submitted herewith as Figure 1 and Figure 2 was obtained in my laboratory under my direct supervision. The following is a description of how the data for each figure was obtained.

Liver Cancer Data: Description for Figure 1

To investigate whether 67LR mediates the anti-liver cancer action of EGCG, we have constructed both 67LR-overexpressing or -downregulating human liver cancer cell line HepG2 by transfecting with the 67LR gene or 67LR-specific siRNA. To elucidate whether the

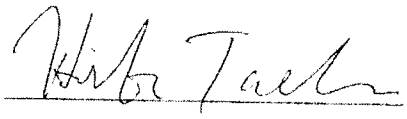
expression of 67LR is enhanced in HepG2 cells transfected with the 67LR gene or is reduced in the cells transfected with the 67LR-specific siRNA, we first examined the expression of 67LR in these transformed cells. Western blot analysis showed that higher expression level of 67LR in the 67LR transfected cells (Figure 1a) or lower level in the 67LR-specific siRNA transfected cells as compared with each control cells transfected with empty vector. Secondly, we examined the binding of EGCG to these cells. The surface plasmon resonance assay demonstrated that 67LR-overexpressing cells have increased EGCG binding to the cell surface (Figure 1b), whereas the 67LR-downregulating cells have reduced the ability to bind EGCG. Thirdly, we examined EGCG sensitivity of these cells for their cell growth. The control cells transfected with empty vector showed no growth inhibition when the cells were treated with EGCG at the concentration of 1 μM, which is physiologically relevant concentration in human after tea drinking (Figure 1c). The 67LR-overexpressing cells treated with the same concentration of EGCG demonstrated considerable inhibition as compared with the control cells (Figure 1c). The control cells transfected empty vector and treated with 5 μM EGCG showed considerable growth inhibition on the other hand, 67LR-downregulating cells treated with the same concentration of EGCG demonstrated no growth inhibition. These results suggested that 67LR is involved in the binding of EGCG to the cell surface and EGCG-mediated growth inhibition of HepG2 liver cancer cells. These data has been published as "67 kDa laminin receptor sensitizes human hepatocytes to tea (−)-polyphenol epigallocatechin-3-O-gallate" in Proceedings of 2004 international conference O-CHA (tea) culture and science, 477-478 (2005), included herewith as Exhibit A.

Breast Cancer Data: Description for Figure 2

To investigate whether 67LR mediates the anti-breast cancer action of EGCG, we have established human breast cancer MCF-7 cells stably expressing reduced 67LR by transfecting with the 67LR-specific siRNA. Western blot analysis showed that lower level in the 67LR-specific siRNA transfected cells as compared with control cells transfected with empty vector (Figure 2a). The control cells transfected with empty vector and treated with 20 μM EGCG showed considerable growth inhibition. On the other hand, 67LR-downregulating cells treated with the same concentration of EGCG demonstrated no growth inhibition (Figure 2b).

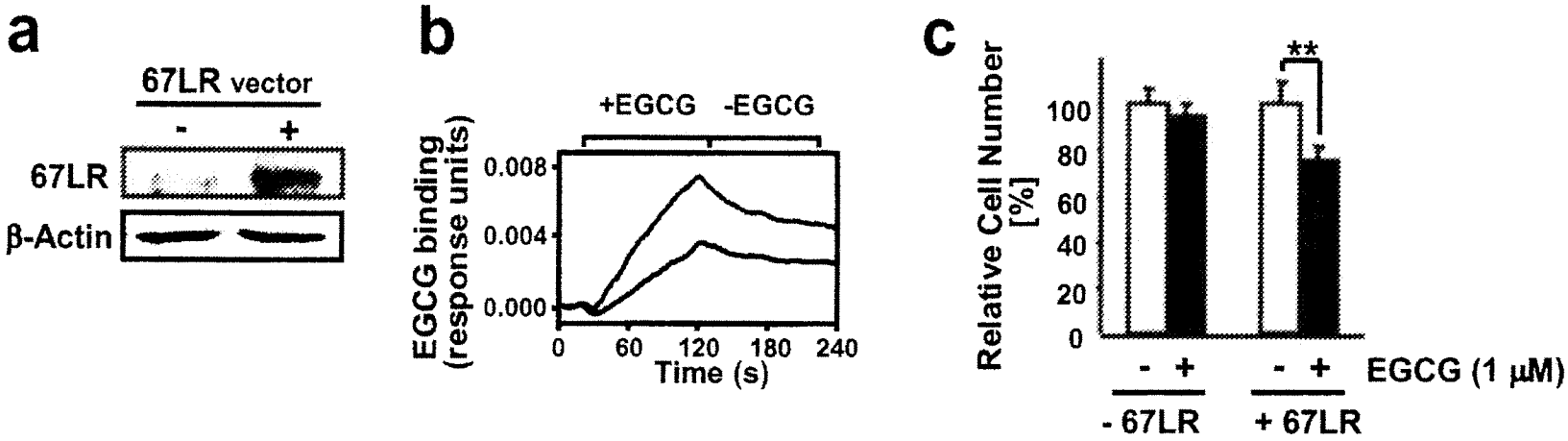
These results suggested that 67LR is involved in the binding of EGCG to the cell surface of and EGCG-mediated growth inhibition of MCF-7 breast cancer cells.

3. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application and any patent issuing thereon.

Date March 10, 2009 Signed 

Hirofumi Tachibana

Anti-Liver cancer (HepG2) action of EGCG is mediated by the 67 kDa laminin receptor



(a) 67 LR and β-actin protein levels from the human hepatoma HepG2 cells transfected with the 67 LR vector (+) or the empty vector (-) .

(b) EGCG binding to the surface of HepG2 cells transfected with the 67 LR vector (red line) or the empty vector (black line) measured by surface plasmon resonance.

(c) HepG2 cells transfected with the 67 LR vector (+ 67LR) or empty vector (- 67LR) were exposed to 1 μM EGCG (black bar) or water (white bar) for 3 days, and the cell numbers were assessed. The data presented is the mean (± s.e.m.) of triplicate experiments (**P<0.01).*

Fig. 1

Exhibit A

67 kDa LAMININ RECEPTOR SENSITIZES HUMAN HEPATOCYTES TO TEA POLYPHENOL (–)-EPIGALLOCATECHIN-3-*O*-GALLATE

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Summary

(–)-Epigallocatechin-3-*O*-gallate (EGCG) is the major polyphenolic compound of green tea. EGCG has been shown to have various biological activities including anti-tumor, anti-inflammation and anti-obesity. However, little is known about the possible target molecules that mediates these activities. Recently, we have identified the 67 kDa laminin receptor (67LR) as a cell surface receptor that mediates the anti-tumor action of EGCG, but it is still unclear whether 67LR mediates other activities of EGCG. In the present study, to investigate whether 67LR mediates the metabolic action of EGCG in human hepatocytes, we have constructed both 67LR-overexpressing or -downregulating human liver cell line HepG2 by transfecting with the 67LR gene or 67LR-specific siRNA. Enhanced expression of 67LR in HepG2 cells increased EGCG binding to the cell surface, whereas the 67LR-downregulated cells have reduced the ability to bind EGCG.

Keywords

Receptor, EGCG, HepG2, Hepatocytes, 67 kDa laminin receptor

Introduction

Green tea is one of the major beverage worldwide, and is known to contain high amount of polyphenolic compounds. The major polyphenolic compound of green tea, (–)-epigallocatechin-3-*O*-gallate (EGCG) has been reported to have various biological activities including anti-tumor, anti-inflammation and anti-oxidation. It is also known that EGCG has anti-obesity due to decreasing cholesterol synthesis and increasing insulin sensitivity. Although the candidate molecule which can mediate these activities of EGCG was unknown, we have recently identified the 67 kDa laminin receptor (67LR) as a cell surface receptor that mediates the anti-tumor action of EGCG. Here, to examine whether 67LR mediates the metabolic action of EGCG in liver, we have constructed both

67LR-overexpressing or -downregulating human liver cell line HepG2.

Results and discussion

To elucidate whether the expression of 67LR is enhanced in HepG2 cells transfected with the 67LR gene or is reduced in the cells transfected with the 67LR-specific siRNA, we first examined the expression of 67LR in these transformed cells. Western blot analysis showed that higher expression level of 67LR in the 67LR transfected cells or lower level in the 67LR-specific siRNA transfected cells as compared with each control cells transfected with empty vector. Secondly, we examined the binding of EGCG to these cells. The surface plasmon resonance assay demonstrated that 67LR-overexpressing cells have increased EGCG binding to the cell surface, whereas the 67LR-downregulating cells have reduced the ability to bind EGCG. Thirdly, we examined EGCG sensitivity of these cells for their cell growth. The control cells transfected with empty vector showed no growth inhibition when the cells were treated with EGCG at the concentration of 1 μM, which is physiologically relevant concentration in human after tea drinking. The 67LR-overexpressing cells treated with the same concentration of EGCG demonstrated considerable inhibition as compared with the control cells (Figure 1A). The control cells transfected empty vector and treated with 5 μM EGCG showed considerable growth inhibition on the other hand. 67LR-downregulating cells treated with the same concentration of EGCG demonstrated no growth inhibition (Figure 1B). These results suggested that 67LR is involved in the binding of EGCG to the cell surface and EGCG-mediated growth inhibition of HepG2 cells.

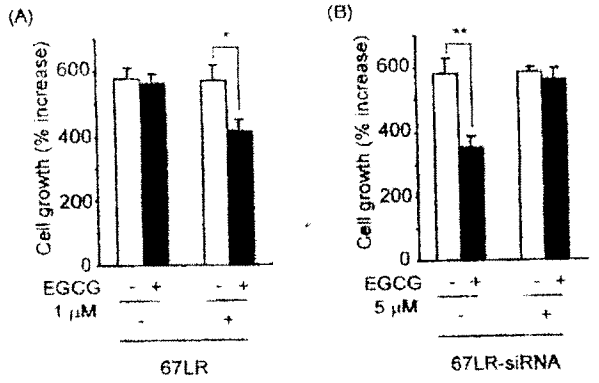
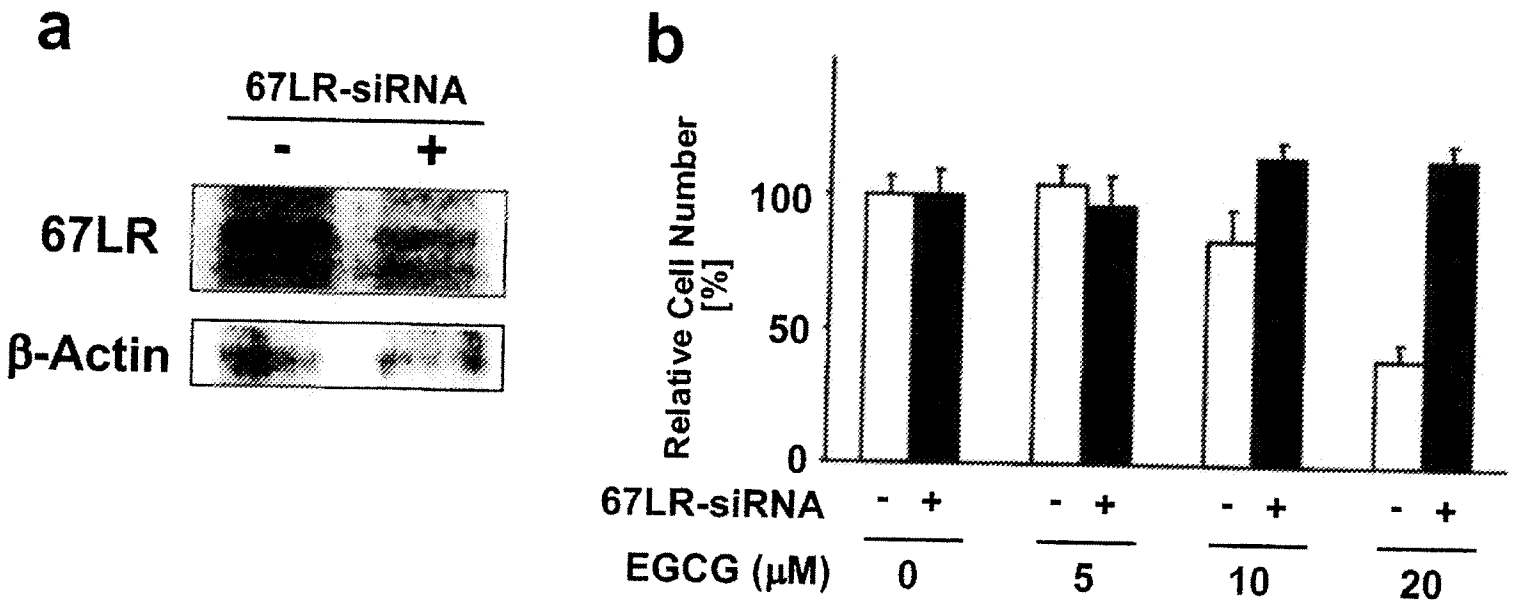


Figure 1 Effect of the 67LR gene or 67LR specific siRNA transfection on EGCG sensitivity. (A) The 67LR-overexpressed cells were treated with or without 1 μM EGCG for 5 days. (B) The 67LR-downregulated cells were treated with or without 5 μM EGCG. The data containing asterisk marks are significantly different from EGCG untreated groups at $p<0.05$* and $p<0.01$** ($n=3$).

References

Tachibana *et al.*, *Nature Struct. Mol. Biol.*, 11, 380, 2004

Anti-Breast cancer (MCF-7) action of EGCG is mediated by the 67 kDa laminin receptor



(a) 67 LR and β-actin protein levels from the human breast cancer MCF-7 cells transfected with the 67LR siRNA vector (+) or the empty vector (-) .

(b) MCF-7 cells transfected with the 67LR siRNA vector (+; black bar) or empty vector (-; white bar) were exposed to EGCG for 3 days, and the cell numbers were assessed.

Fig. 2